Cooperativity in F-Actin: Chemical Modifications of Actin Monomers Affect the Functional Interactions of Myosin with Unmodified Monomers in the Same Actin Filament

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ABSTRACT We have chemically modified a fraction of the monomers in actin filaments, and then measured the effects on the functional interaction of myosin with unmodified monomers within the same filament. Two modifications were used: (*a*) covalent attachment of various amounts of myosin subfragment-1 (S1) with the bifunctional reagent disuccinimidyl suberate and (*b*) copolymerization of unmodified actin monomers with monomers cross-linked internally with 1-ethyl-3-(dimethylaminopropyl)-carbodiimide. Each of these modifications abolished the interaction of the modified monomers with myosin, so the remaining interactions were exclusively with unmodified monomers. The two modifications had similar effects on the interaction of actin with myosin in solution: decreased affinity of myosin heads for unmodified actin monomers, without a change in the V_{max} of actin-activated myosin ATPase activity. However, modification (*b*) produced much greater inhibition of actin sliding on a myosin-coated surface, as measured by an in vitro motility assay. These results provide insight into the functional consequences of cooperative interactions within the actin filament.

INTRODUCTION

The actin filament (F-actin) is a two-stranded helical polymer of globular actin monomers; interaction with myosin in the presence of ATP results in activation of myosin ATPase and sliding of the actin filament along myosin heads. Spectroscopic studies have shown that the binding of myosin heads can produce a nonlinear relationship between the extent of spectral changes in actin-attached spin or fluorescent labels and the fraction of actin monomers having bound heads, with the effect saturating at much less than one head/actin monomer (1–4). These results suggest that the interaction of actin with myosin involves cooperative structural changes in F-actin propagated through intermonomer bonds along the filament.

In the present study, we have taken a complementary approach in studying cooperativity within F-actin. We have introduced structural changes by chemically modifying a fraction of monomers within the actin filament, then determined the effects on the functional interaction of neighboring (unmodified) monomers with myosin. We measured the effects on myosin binding and actomyosin ATPase activity in solution and on the sliding movement of actin filaments on a myosin-coated surface. In order to simplify the interpretation, we used chemical modifications of actin monomers that completely inhibit the interaction of actin monomers with myosin, so that any remaining interaction must be with unmodified monomers.

We used two different methods of actin modification. One method was incorporation of modified monomers into actin filaments during polymerization, which was achieved by copolymerization of unmodified actin monomers with actin monomers that had been internally cross-linked with EDC (1-ethyl-3-(dimethylaminopropyl)carbodiimide). These EDC-cross-linked monomers do not activate myosin ATPase and do not bind myosin in the presence of ATP (5). In the other method, actin was polymerized and combined with myosin subfragment-1 (S1), and then the bound S1 was covalently cross-linked to actin with the bifunctional reagent disuccinimidyl suberate (DSS). We used DSS because, in contrast to EDC, when this cross-linker is reacted with actin in the absence of myosin, it does not affect the motility of F-actin and does not decrease actin's affinity for myosin (5). Therefore, in this DSS-cross-linked acto-S1 preparation, we could assume that unoccupied monomers, i.e., monomers without cross-linked S1, are equivalent to unmodified monomers with respect to their interaction with myosin. The effects of these modifications on the functional interactions of myosin provide insight into the functional consequences of intermonomer interactions in actin filaments.

MATERIALS AND METHODS

Protein preparation

Unless otherwise indicated, all preparations and procedures were carried out at 4°C. Actin was extracted from acetone-dried muscle powder and purified by polymerization with 30 mM KCl; on polyacrylamide gel electrophoresis such preparations were free from regulatory proteins (6). Heavy meromysin (HMM) and S1 were obtained by α-chymotryptic digestion of myosin from rabbit skeletal muscle as described by Weeds and Pope (7) and Weeds and Taylor (8), lyophilized in 0.1 M sucrose, and stored at −20°C.

Concentration of unlabeled protein was measured by ultraviolet absorption, assuming the following values of molar extinction coefficient of 1% protein solutions: for S1 A_{280} = 7.5 cm^{-1}, M_r = 110,000; for HMM A_{280} = 0.647, M_r = 350,000; and for actin A_{290} = 0.63, M_r = 42000; concentration of labeled proteins was determined using BioRad protein assay with standards made from unlabeled S1 and actin.

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Labeling S1 with IATR

IATR (iodoacetamidotetramethylrhodamine), freshly dissolved in dimethylsulfoxide was added to 3 mg/ml of S1 in 50 mM KCl, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), pH 8.0, at a molar ratio of 4:1. After a 17-h incubation at 4°C, the reaction was terminated with 1% β-mercaptoethanol, and labeled S1 was clarified by 15-min ultracentrifugation at 70,000 rpm (Beckman TL 100 ultracentrifuge). Unreacted dye was removed on a Sephadex G25 column equilibrated with 50 mM KCl, 20 mM Hepes (pH 8.0); the extent of labeling was 1.8–2.2 mol of IATR/mol S1, assuming a molar extinction coefficient for IATR at 554 nm of 5×10^4 M^{-1} cm^{-1}.

Modification of S1 with NEM

3 mg/ml of S1 in 20 mM Hepes, pH 7.0, and 50 mM KCl was incubated for 40 min at 25°C with 20 times molar excess of *N*-ethylmaleimide (NEM). The reaction was terminated with 1% β-mercaptoethanol, unreacted NEM was removed by dialysis against 20 mM Hepes, pH 8.0, and NEM-S1 was clarified by 15-min ultracentrifugation at 70,000 rpm.

Cross-linking of F-actin with NEM-S1 and IATR-S1

Modified S1 was mixed with 0.21 mg/ml of F-actin at various molar ratios in 50 mM KCl, 2 mM $MgCl_2$, 20 mM Hepes (pH 8.0). The cross-linking reaction was initiated by addition of DSS, freshly dissolved in dimethylsulfoxide, at a 10:1 molar ratio to actin. A 40- or 60-min incubation at 25°C resulted in cross-linking of up to 0.5 mol of modified S1/mol of actin. A further addition of DSS, followed by a 40-min incubation, increased the extent of cross-linking to about 0.8 mol of modified S1/mol of actin. The reaction was terminated by the addition of 0.4 M KCl, 2.5 mM $MgCl_2$, 2 mM ATP, 20 mM Tris (pH 8.0). Uncross-linked S1 was then removed by sedimenting the cross-linked complex for 1-h at 48000 rpm (L-7 ultracentrifuge; Beckman Instruments, Palo Alto, CA). Cross-linking of F-actin alone was carried out under identical conditions. The pellets of DSS-cross-linked acto-NEM-S1, acto-IATR-S1, and F-actin were homogenized in 2 mM $MgCl_2$, 0.1 mM ATP, 20 mM Tris (pH 8.0), and dialyzed against this solution. The amounts of cross-linked NEM-S1 and IATR-S1 were calculated by measuring the concentration of S1 remaining in the supernatant after ultracentrifugation, using the Bio-Rad protein assay calibrated against known concentrations of NEM-S1 or IATR-S1. Calculation of the amount of cross-linked IATR-S1 by measurement of absorbance of initial samples and supernatants at 554 nm gave the same results.

Copolymers

Copolymerization of unmodified actin monomers with monomers labeled with IATR and cross-linked with EDC was carried out as described previously (5).

Electron microscopy

Protein samples put on bare grids were washed with several drops of 2 mM $MgCl_2$, 0.2% NaN_3, 5 mM potassium phosphate (pH 7.0), without or with 0.5 mM ATP, then negatively stained with 1% uranyl acetate containing 20 μg/ml bacitracin. Electron micrographs were taken with a JEOL 200ES electron microscope, as described previously (9).

Binding of HMM and S1 to F-actin

For determination of the amounts of HMM bound to actin in the presence of ATP, various concentrations of actin were preincubated at 25°C in buffer containing 2 mM ATP, 2.5 mM $MgCl_2$, 20 mM Tris (pH 8.0), then 0.035 mg/ml HMM was added and samples were sedimented at 70,000 rpm (Beckman TL-100 ultracentrifuge) at 25°C for 15 min. For determination of the amounts of S1 bound to actin in the absence of ATP, various concentrations of S1 were preincubated with a constant concentration of actin for 10–15 min at 25°C in 50 mM KCl, 2.5 mM $MgCl_2$, 20 mM Tris (pH 8.0), and then samples were centrifuged at 70,000 rpm (Beckman TL-100) at 25°C for 15 min. The concentration of unbound HMM or S1 was determined by measurement of NH_4/EDTA-ATPase activities of the supernatant after ultracentrifugation, as described previously (5).

ATPase measurements

Actin-activated MgATPase of HMM and S1, and MgATPase of the cross-linked acto-NEM-S1 or acto-IATR-S1 complexes, was measured in the presence of 2.5 mM $MgCl_2$, 2 mM ATP, 20 mM Tris (pH 8.0), at 25°C. The reaction was initiated by the addition of ATP and terminated by the addition of 1.3% SDS. P_i was determined by the Fiske-Subbarow method (10). Depolymerization of F-actin at low concentration (≤0.1 mg/ml) was prevented by addition of equimolar amounts of phalloidin. The Ca-ATPase activity of S1 was measured in the presence of 50 mM KCl, 10 mM $CaCl_2$, 2 mM ATP, 20 mM Hepes (pH 8.0), at 25°C, with 0.15 mg/ml of unmodified S1 or IATR-S1, or 0.5 mg/ml of NEM-S1. P_i was determined by the Fiske-Subbarow method. The potassium/EDTA-ATPase activity of S1 was measured in the presence of 0.5 M KCl, 10 mM ATP, 20 mM Hepes (pH 8.0), at 25°C, with 0.01 mg/ml of unmodified S1, 0.3 mg/ml of NEM-S1, or 0.02 mg/ml of IATR-S1. To prevent denaturation and/or adsorption of diluted S1 on the surface of the glass, 0.1 mg/ml bovine serum albumin was added to the incubation mixture. P_i was determined by the malachite green method (11).

Motility assay

The sliding velocity of fluorescent actin on an HMM-coated surface was measured essentially as described previously (5). 10 μl of 0.5 mg/ml HMM freshly dissolved in buffer (20 mM Tris, pH 8.0) was spread on Sigmacote-coated cover glass. After about 1 min, unattached HMM was removed by a buffer wash containing 2.5 mM $MgCl_2$, 0.2 mM ATP, 1% β-mercaptoethanol, 10 mM Tris (pH 8.0). Fluorescent F-actin, labeled with phalloidin-rhodamine (IATR-phalloidin) was added. Fluorescent actin filaments were observed in the same buffer wash containing additionally an oxygen-removing system: 4.5 mg/ml glucose, 216 μg/ml glucose oxidase, and 36 μg/ml catalase; for better protection of the sample from damage by light, an ND filter (50) was always inserted between the light source and the sample. Images were recorded on videotape with a high-sensitivity camera (Ikegami SIT CTC 9000) and a video recorder (SVHS MacLord HiFi GT4).

The criterion to distinguish between motile or nonmotile filaments was as follows: A filament's speed of sliding was determined by observing the position of its end for at least 5 s. If this position did not change significantly, the filament was considered nonmotile; if the position changed significantly, the filament was considered motile. Since a 1-μm distance corresponded to 5 mm on the recorded image on the video screen, nonmotile filaments had velocities less than 0.2 μm/s. When more than 50% of all visible filaments in a field were motile, the filaments moved smoothly and their speeds were very homogenous. However, when the fraction of motile filaments decreased, the movement becomes less homogenous: some of the filaments still moved smoothly, but others were observed to undergo intermittent sliding, punctuated by brief pauses. These intermittently sliding filaments were still considered motile, and their mean velocities were measured over a period of several seconds, just as for the smoothly sliding filaments. As the fraction of smoothly sliding filaments decreased, the standard deviation of velocities increased. This approach produced a higher estimate of the fraction of motile filaments than would be obtained if intermittently sliding filaments were considered nonmotile, but gave us a sensitive measure of complete inhibition of movement. The same method of defining motile and nonmotile filaments was applied in our previous work (5).

Reagents

IATR and phalloidin rhodamine were obtained from Molecular Probes; EDC, glucose oxidase, catalase, and ATP were obtained from Sigma; all

other reagents were of the highest grade of purity and were supplied by Nakarai Chemicals (Kyoto, Japan) or Sigma (St. Louis, MO).

RESULTS

Characterization of DSS-cross-linked acto-S1 complexes

As shown below, direct proof that actin with covalently attached myosin heads can slide was obtained by observation of actin filaments to which IATR-S1 was cross-linked with DSS. The labeling of S1 with IATR was done before cross-linking and resulted in an increase of Ca-ATPase and decrease of EDTA-ATPase activities of S1 (Table 1), characteristic for modification of Cys-707 (SH1) on S1 (12). Excessive reaction of S1 with NEM inhibited both ATPase activities (Table 1), indicating the blocking of both Cys-797 (SH1) and Cys-697 (SH2); this modification has been shown to prevent any effect of ATP on S1 bound to actin (13). When IATR-S1 and NEM-S1 were cross-linked to F-actin with DSS, the MgATPase activity of cross-linked complexes was almost completely inhibited, in comparison with the activity of unmodified S1 bound to actin (Table 2). Table 3 shows the binding of free S1 to cross-linked complexes in the absence of ATP. The amounts of bound S1 were comparable with the amounts of unoccupied monomers in the complexes, calculated as the difference between total concentration of actin and concentration of covalently attached heads; this shows that cross-linked IATR-S1 and NEM-S1 effectively blocked myosin binding sites on the occupied monomers, but not on the unoccupied monomers.

Electron microscopy of DSS-cross-linked acto-S1 complexes

Fig. 1 shows electron micrographs of DSS-cross-linked acto-IATR-S1 complexes containing increasing amounts of cross-linked heads; at a given average number of cross-linked IATR-S1, all actin filaments were decorated to a similar extent, and IATR-S1 was attached in a normal arrowhead pattern. It is very important that the samples did not contain mixtures of fully decorated and sparsely decorated or naked filaments. In the absence of ATP, cross-linked acto-IATR-S1 and acto-NEM-S1 complexes had the same appearance. Addition of ATP to DSS acto-NEM-S1 complex did not change the shape of cross-linked heads; the arrowhead pattern remained, showing directly that cross-linked NEM-S1 is strongly bound to actin, not only in the absence but also in the presence of ATP (Fig. 2 *a*). However, in the case of the DSS-cross-linked acto-IATR-S1 complex, addition of ATP changed the shape of cross-linked heads; they became small and round, and did not form regular arrowhead structures (Fig. 2 *b*).

TABLE 1 The effect of modification of S1 with IATR and NEM on calcium and potassium/EDTA-ATPase

Sample	Ca-ATPase	Potassium/EDTA-ATPase
S1	3.47 ± 1 (5)	9.45 ± 0.9 (4)
IATR-S1	4.9 ± 1.1 (5)	3.6 ± 0.7 (4)
NEM-S1	0.5 ± 0.4 (3)	0.02 ± 0.02 (2)

ATPase rates are expressed as micromoles of P_i/micromoles of heads/s. The values indicate mean and standard deviation. The number of measurements is given in parentheses.

TABLE 2 Mg-ATPase activity of DSS-crosslinked acto-NEM-S1 and acto-IATR-S1 complexes

Sample	Moles of NEM-S1 or IATR-S1 per mole of actin	V_{max}
		(%)
DSS-acto-NEMS1	0.45–0.62	0.12 ± 0.1 (3)
DSS-acto-IATRS1	0.20–0.62	4 ± 2 (6)

100% corresponds to the V_{max} of intact acto-S1 (20 ± 2 μmol of P_i/μmol of S1/s). The values indicate the mean and standard deviation. The number of measurements is given in parentheses.

TABLE 3 Binding of S1 to DSS-crosslinked acto-NEM-S1 and acto-IATR-S1 complexes in the absence of ATP

Sample	Fraction of unoccupied monomers in F-actin	Bound S1, mol/mol of total actin in the complex
DSS-acto-NEM-S1	0.66 ± 0.15 (6)	0.60 ± 0.20 (6)
DSS-acto-IATR-S1	0.66 ± 0.06 (4)	0.46 ± 0.04 (4)
DSS-actin	1	1.16
Intact actin	1	1.2

Molar ratio of added free S1 to the amount of crosslinked NEM-S1 or IARS1 varied from 3 to 10. The values indicate mean and standard deviation, and the values in parentheses indicate the number of measurements.

Electron micrographs of EDC-cross-linked actin and copolymers

Fig. 3 shows electron micrographs of unmodified actin, EDC-cross-linked actin, and copolymers made from cross-linked and unmodified actin, containing 70 and 50% of cross-linked monomers. EDC-cross-linked monomers formed very short filaments; these filaments had a tendency to form loose aggregates, but dilution to low concentration dispersed the aggregates. Under conditions of the motility assay (about 0.003 mg/ml of actin) only single filaments were observed. After copolymerization with unmodified actin, filaments did not aggregate and their length gradually increased, but these filaments of copolymers still were shorter than the filaments of unmodified actin.

Interaction of modified actin filaments with free myosin heads in solution

The concentration of DSS and the time of cross-linking were adjusted to minimize the effect of DSS on actin alone: treatment of actin with DSS in the absence of S1, under the same conditions used to prepare the complexes, did not affect activation of myosin ATPase (Fig. 4). The experiments with DSS-cross-linked acto-NEM-S1 (IATR-S1) complexes and copolymers of EDC-modified and unmodified actin were focused on examination of the interaction between unoccupied

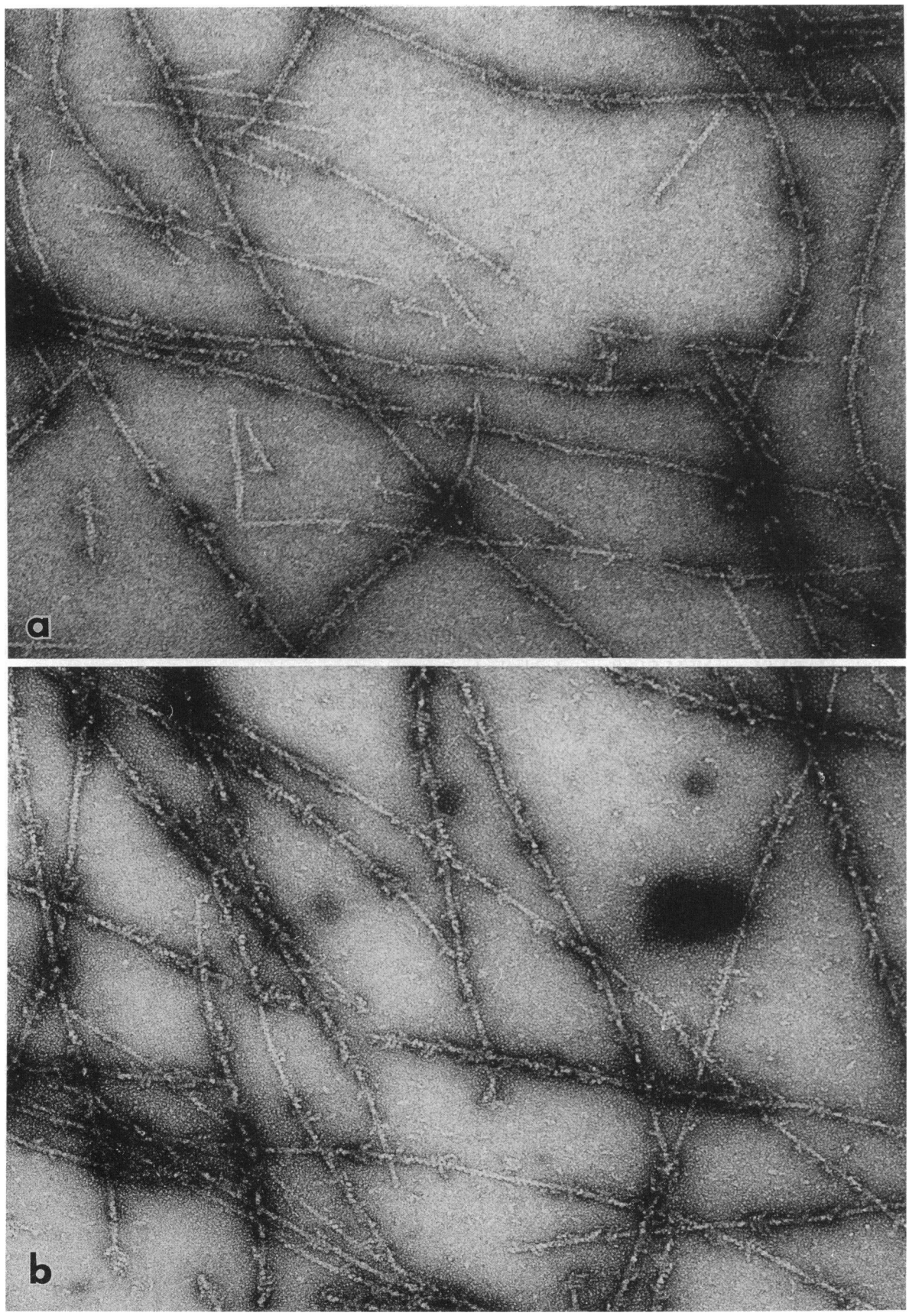

FIGURE 1 Electron micrographs of DSS-cross-linked acto-IATR-S1 complexes in the absence of ATP. The percent of cross-linked IATR-S1 and of sliding filaments were (*a*) 5 and 56%, (*b*) 40 and 48%, (*c*) 68 and 0%. 100% corresponds to a 1:1 molar ratio of cross-linked IATR-S1 to actin and all filaments seen in the field sliding. Magnification, 250,000 ×.

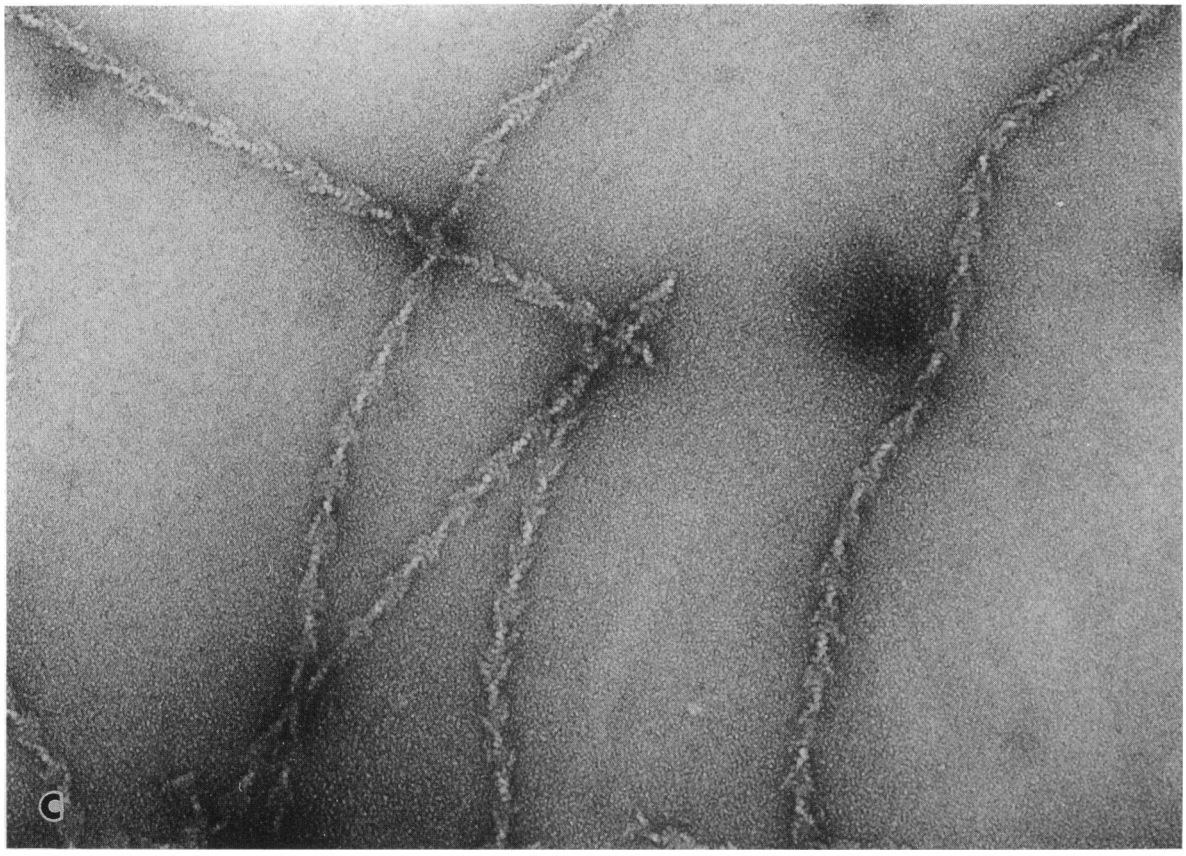

FIGURE 1 *Continued.*

TABLE 4 Activation of Mg-ATPase of HMM and S1 by unmodified actin, copolymers of unmodified and EDC-crosslinked monomers and by DSS-crosslinked acto-NEM-S1 complexes

		$1/K_m \times 10^5$ M^{-1}		
Subfragment	V_{max} s^{-1}	Unmodified actin	Copolymer	DSS-acto-NEM-S1
HMM	18.3 ± 2.1 (6)	1.43 ± 0.48 (6)	0.51 ± 0.04 (4)	0.82 ± 0.22 (4)
S1	19.5 ± 1.2 (6)	0.93 ± 0.13 (6)	0.43 ± 0.07 (3)	0.45 ± 0.17 (4)

The fraction of EDC-crosslinked actin in copolymers varied from 40 to 54% and molar ratio of covalently bound NEMS1 to actin varied from 0.37 to 0.52.

or unmodified monomers in modified filaments with added HMM or S1. V_{max} and K_m (the apparent K_m with respect to actin, sometimes designated K_{ATPASE}) of actin-activated myosin ATPase were obtained by measurement of ATPase rates versus concentration of added HMM or S1 at constant concentrations of unoccupied monomers in cross-linked complexes and of unmodified monomers in copolymers, obtained as the difference between total concentration of actin and concentration of covalently bound NEM-S1 (IATR-S1) or EDC-cross-linked monomers. This approach seems most suitable for characterization of the effect of modified monomers on interaction of myosin with remaining monomers. In the alternative approach, extrapolation of the data to infinite concentration of actin, only a small fraction of actin monomers can interact with S1 at high concentrations, possibly obscuring the effects of actin modification. In control experiments, we confirmed that in the case of unmodified and DSS-cross-linked F-actin, the rate of ATP hydrolysis expressed/mol of actin at infinite S1 is the same as the rate expressed/mol of S1 at infinite actin. This result agrees with data of Wagner and Weeds (14), who demonstrated that the two values are equal at 20°C and significant differences between them are found only at 5°C.

Fig. 5 shows one example of the results of actin-activated S1 ATPase, and Table 4 summarizes results obtained in 12 experiments. Neither of the two actin modifications affected the V_{max} of actin-activated S1 ATPase, but the values of K_m for modified filaments were about twice that of unmodified actin. Data obtained for acto-IATR-S1 complex were the same as those for the acto-NEM-S1 complex, and ATPase rates were the same whether HMM or S1 were used. Control experiments confirmed that an increase of the amount of

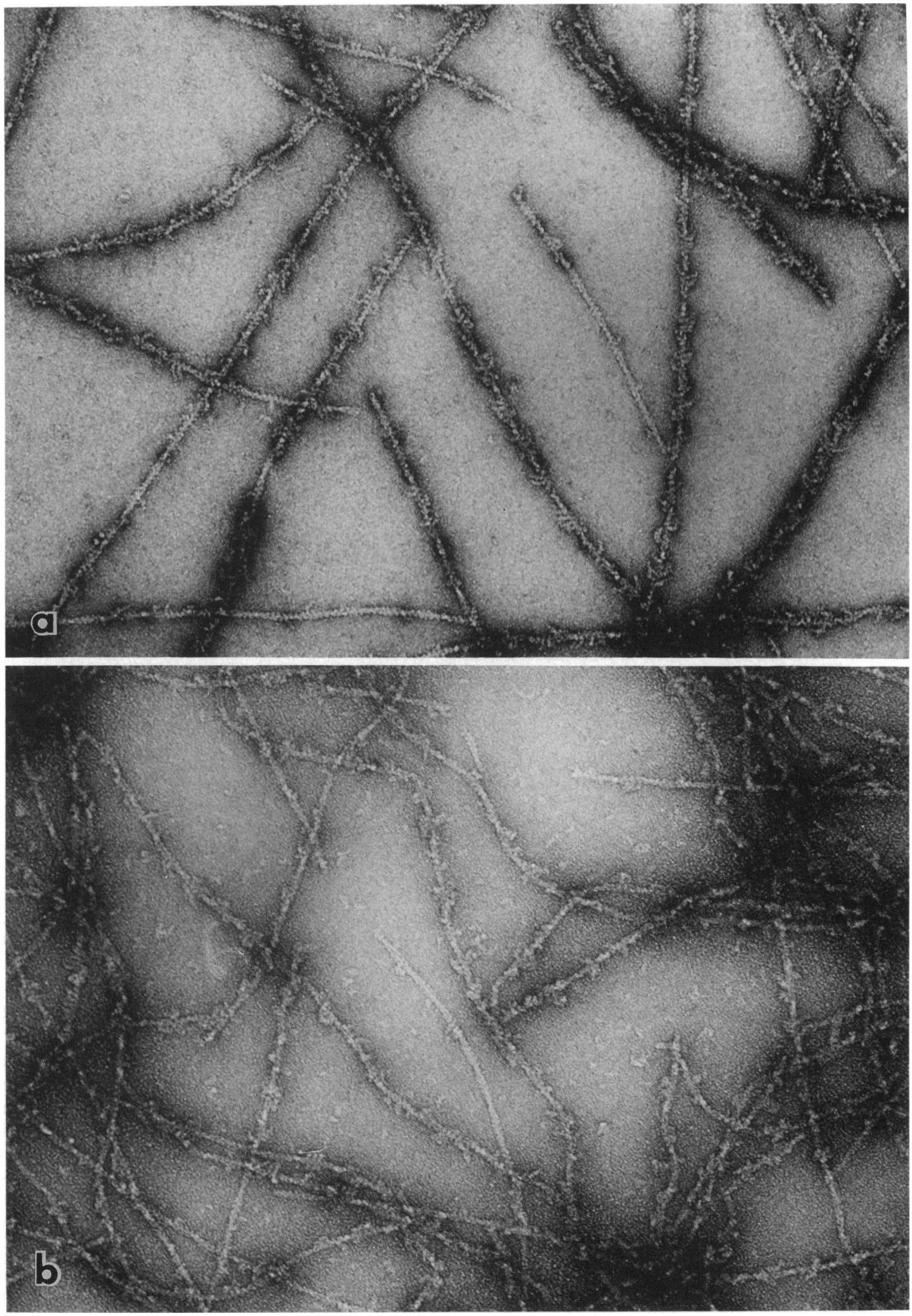

FIGURE 2 Electron micrographs of DSS-cross-linked complexes in the presence of ATP: (*a*) acto-NEM-S1, (*b*) acto-IATR-S1. The fraction of cross-linked heads were 0.5 (*a*) and 0.45 (*b*). Magnification, 250,000 ×.

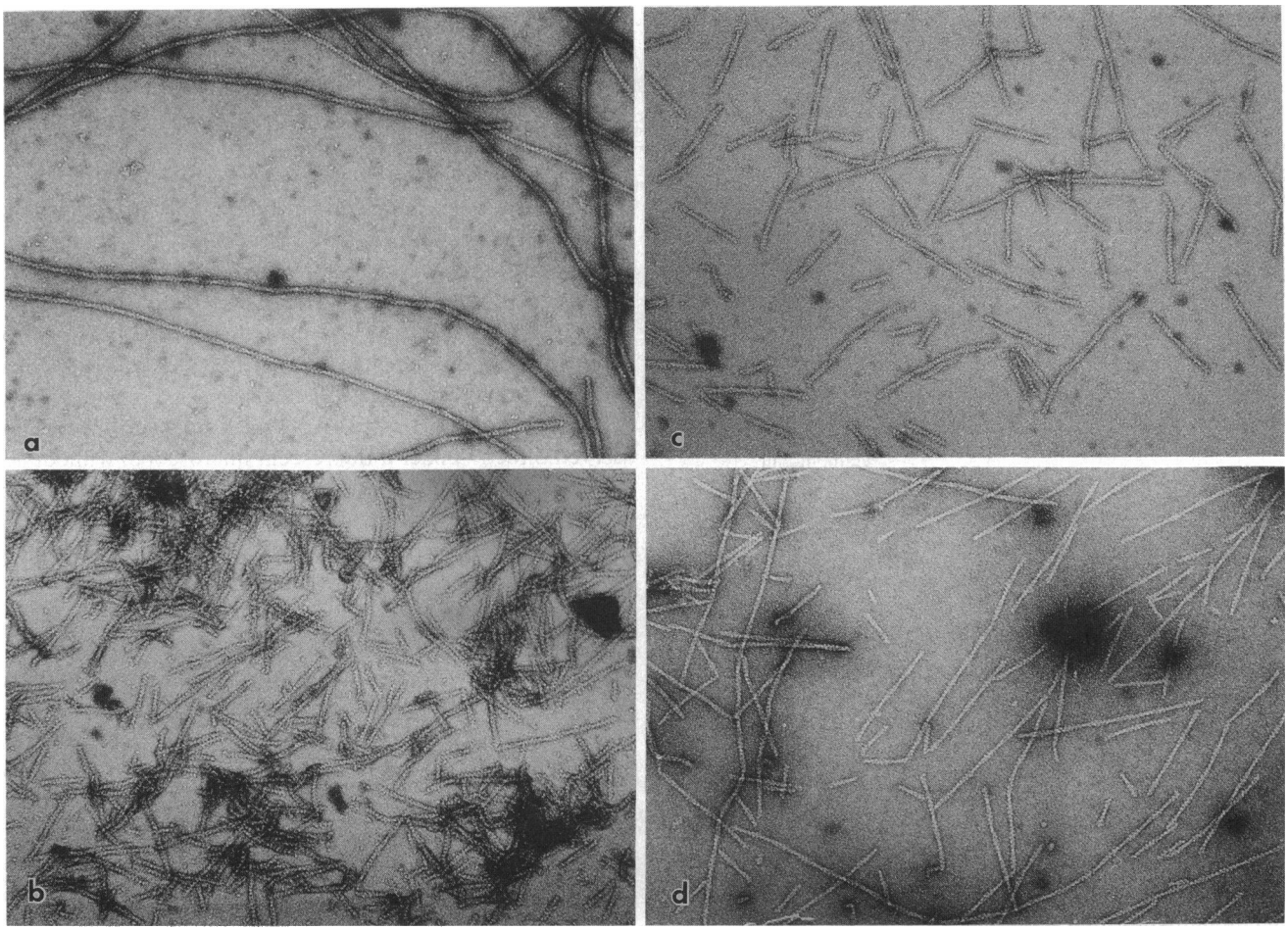

FIGURE 3 Electron micrographs of (*a*) unmodified F-actin, (*b*) actin filaments made of EDC-cross-linked monomers, (*c*) copolymers containing 70% of EDC-cross-linked monomers, and (*d*) copolymers containing 50% of EDC-cross-linked monomers. Magnification, 250,000 ×.

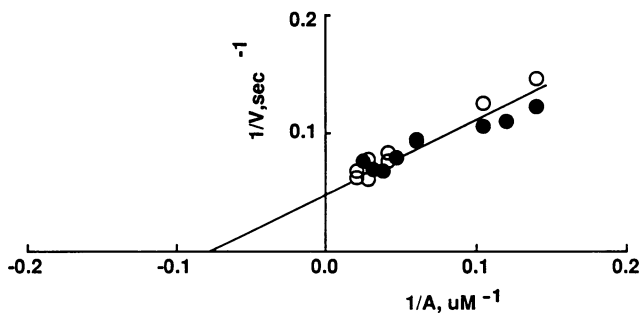

FIGURE 4 Activation of myosin ATPase by unmodified (○) and DSS-modified actin (●). Concentration of S1 was 0.03–0.06 mg/ml. The ATPase rate is expressed as μmol of P_i/μmol of S1/s.

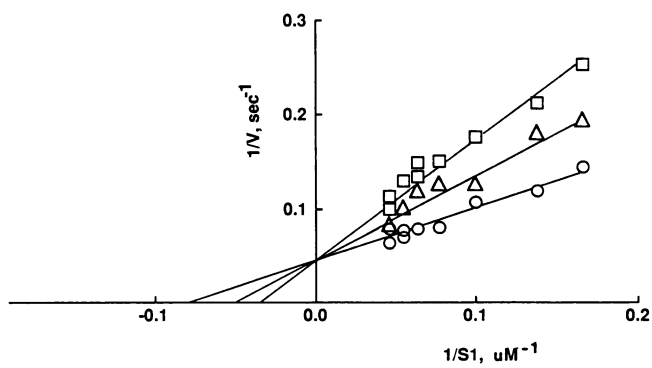

FIGURE 5 Activation of S1 ATPase by unmodified actin (○), unoccupied monomers in DSS-cross-linked acto-NEM-S1 complex (△), and unmodified monomers in EDC-cross-linked and unmodified actin (□). The concentration of actin was 0.1 mg/ml. The ATPase rate V is expressed as μmol of P_i/μmol of actin monomers/s.

cross-linked NEM-S1 in the complex to 0.86 mol of NEM-S1/mol of actin still did not change the V_{max} of actin-activated HMM ATPase.

The results of direct measurement of binding of HMM to unmodified and modified actin in the presence of ATP, plotted against the concentration of unoccupied or unmodified monomers, are shown in Fig. 6. These results are consistent with the results of actin-activated myosin ATPase: the presence of modified monomers in actin filament clearly decreases the binding of HMM to unmodified or unoccupied monomers. Experiments with DSS-cross-linked complexes having various amounts of covalently attached NEM-S1

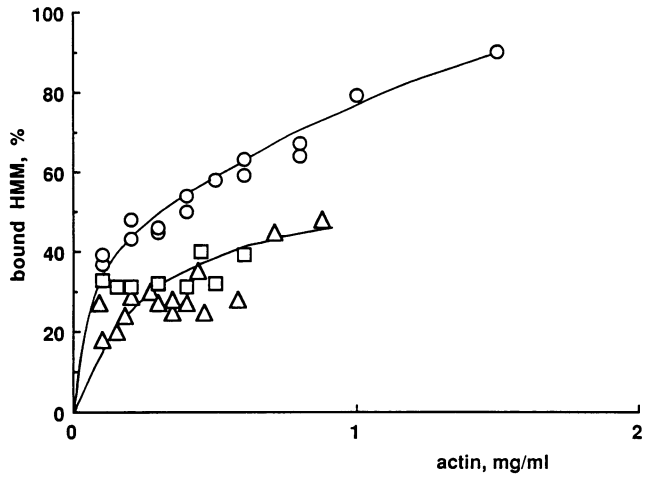

FIGURE 6 Binding of HMM in the presence of ATP to unmodified actin (○), unoccupied monomers in DSS-cross-linked acto-NEM-S1 complex (△), and to unmodified actin in copolymers of EDC-cross-linked and unmodified monomers (□).

show that a significant (10 ± 2%) decrease in the extent of binding of HMM occurs already at 0.11 ± 0.03 cross-linked NEM-S1/actin monomers.

In vitro motility assay

The fraction (Fig. 7) and speed (Fig. 8) of sliding actin filaments was decreasing with the increase in fraction of modified monomers which cannot interact with myosin. This decrease showed nonlinear dependence on the amount of modified monomers and, additionally, the data obtained for copolymers of unmodified and EDC-cross-linked monomers could not be superimposed with data obtained for complexes of covalently cross-linked acto-NEM-S1 (or acto-IATR-S1) complexes. In the case of copolymers of unmodified and EDC-cross-linked monomers, the fraction and speed of sliding filaments were not affected until the fraction of cross-linked monomers was lower than 30%; then both gradually decreased (Figs. 7 and 8). This result is the same as previously reported, when the motility assay was carried out in the presence of 25 mM KCl (5). The only effect of the decrease in [KCl] was a decrease in the length of sliding filaments, due to their fragmentation. Cross-linking myosin heads to F-actin had a less pronounced effect on sliding. The speed of sliding of cross-linked acto-NEM-S1 and acto-IATR-S1 complexes remained the same as that of unmodified actin until the fraction of modified monomers surpassed 50%, then sharply decreased to zero (Fig. 8). As in the case of copolymers or unmodified actin, the change in KCl concentration affected only the length of sliding filaments.

DSS-cross-linked acto-IATR-S1 complexes were observed directly under a fluorescent microscope, without additional labeling of actin with phalloidin-rhodamine. Experiments using an image analysis system did not detect a significant difference in brightness of sliding and nonsliding filaments, indicating that they have similar amounts of cross-linked IATR-S1.

We used DSP (dithiobissuccinimidyl propionate), a cleavable analog of DSS, to demonstrate that the inhibition of sliding movement, due to covalent attachment of S1, is reversible. The filaments of DSP cross-linked acto-IATR-S1 complexes, containing about 70% modified actin monomers, were clearly seen under a fluorescence microscope without labeling with phalloidin-rhodamine; this observation had to be done in the absence of β-mercaptoethanol to avoid cleavage of DSP. Addition of 2% β-mercaptoethanol (to reverse the cross-linking) resulted in a gradual decrease of fluorescence intensity of filaments due to detachment of IATR-S1, and this was accompanied by the appearance of increasing amounts of sliding filaments.

DISCUSSION

The above-described actin modifications have allowed us to study directly the effects of inactive actin monomers on the

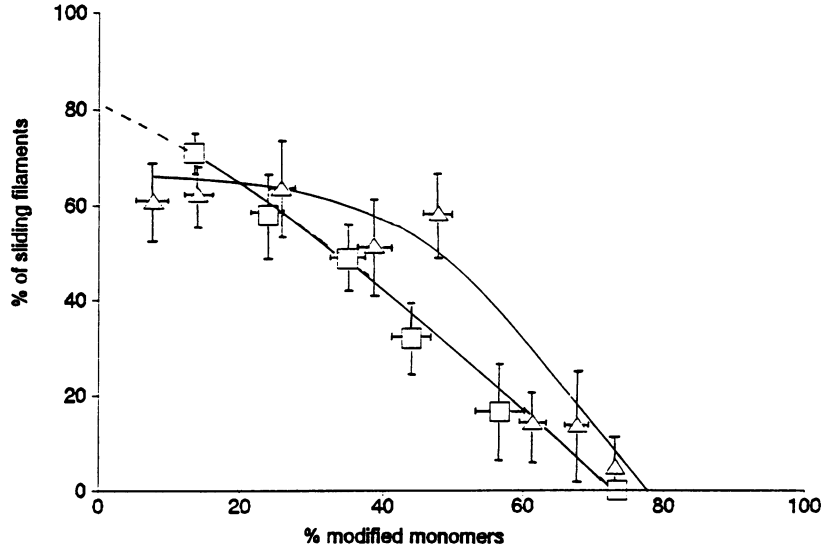

FIGURE 7 The effect of the fraction of modified monomers on the fraction of sliding filaments. EDC-cross-linked monomers in copolymers (□); DSS-cross-linked NEM-S1 and IATR-S1 (△). The fraction of sliding filaments was calculated by subtracting two video frames of recorded image by IBAS image processing system and expressed as the percentage of total amount of filaments seen on the first frame. For each sample, several hundreds of filaments recorded on four or five different fields was counted. Each point represents the average sliding velocity of actin (four to six experiments) and error bars represent standard deviation from the average. In the case of unmodified actin the amount of sliding filaments was 82–94% of the total amount counted in the frames. The data for DSS-cross-linked acto-NEM-S1 and acto-IATR-S1 represent results obtained with 16 preparations, and the data for copolymers represent results obtained with eight preparations.

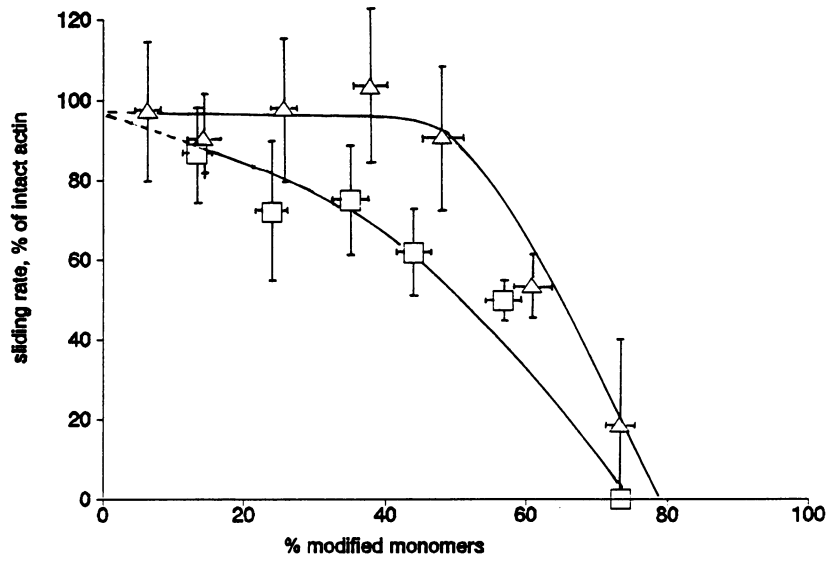

FIGURE 8 The effect of the fraction of modified monomers on the rate of sliding of actin filaments. EDC-cross-linked monomers in copolymers (□); DSS-cross-linked NEM-S1 and IATR-S1 (△). The rate of sliding was calculated as the average rate of 10–15 sliding filaments, and each point represents the average rate obtained in four to six experiments; error bars are standard deviations from the mean. 100% correspond to the sliding rate of unmodified actin (1–2 μm/s, depending on the experiment). Sliding rates and the fraction of sliding filaments were obtained from the same samples.

interaction of neighboring monomers with myosin. The presence of monomers cross-linked with EDC or covalently bound to NEM-S1 (IATR-S1) did not affect V_{max} of myosin ATPase by neighboring monomers, but decreased their apparent affinity ($1/K_m$) for myosin by about half. Similar inhibitions of affinity without changes in V_{max} have been observed for nonmuscle actins and several kinds of chemically modified or cleaved actins (15, 16). This is not surprising, since the actin monomer has at least two regions that bind the myosin heads and the actin-binding and ATP-binding sites on the myosin head are clearly separated (17–19).

Since DSS and EDC react with lysine and carboxyl groups (17, 20), some decrease of myosin affinity could originate from the change of total electrostatic charge of the filaments (21). However, modification of unoccupied monomers in DSS-cross-linked acto-NEM-S1 (or IATR-S1) does not seem to contribute to the decrease of myosin affinity, since modification of pure actin with DSS either increases (5) or leaves unchanged (Fig. 4) actin's affinity for myosin. The possibility of steric inhibition of binding of free heads to unoccupied monomers in cross-linked complexes is rather low: free S1 saturates unoccupied myosin binding sites in the absence of ATP (Table 3) and in the presence of ATP (Fig. 6), binding of myosin heads was the same to DSS-cross-linked acto-IATR-S1 as to acto-NEM-S1, although the addition of ATP affected the shape of cross-linked IATR-S1 but not that of NEM-S1. Furthermore, copolymerization of EDC-modified and unmodified monomers also decreased affinity of free myosin heads for unmodified monomers, and the extent of the decrease was similar to or even slightly higher than in the case of cross-linked acto-NEM-S1 complexes, even though we do not expect any steric hindrance from EDC-modified monomers.

The decrease of affinity of myosin heads for unmodified monomers in our modified actin preparations could directly reflect the often discussed (1–4, 16, 22) cooperative nature of F-actin. We define here cooperativity as the transfer of structural (functional) changes induced in some monomers through intermonomer bonds to neighboring monomers in the filament and also the transfer of structural changes induced in one region of the monomer to another region of the same monomer through the polypeptide chain. Cross-linking actin monomers with EDC probably modifies residues around the amino and carboxyl termini, both of which are located in subdomain 1 of the actin monomer (14, 23–25). Since this subdomain contains bonds with monomers along both the genetic and long-pitch helices (24–26), incorporation of EDC-cross-linked monomers probably alters bonds with two or three unmodified monomers. Subdomain 1 probably also contains myosin binding sites (24, 25), although the manner of binding of myosin to actin (e.g., whether one head binds to one or two actin monomers), is not yet fully explained. Studies on rigor binding of actin to myosin in solution led to the conclusion that myosin binds to actin at a 1:1 ratio of heads to actin (27), but recently the possibility of binding of S1 to actin with a stoichiometry of 1:1 or 1:2, depending on conditions of preparation of the complex, has been discussed (28). Our data are compatible with binding of S1 to actin with stoichiometry 1:1: we were able to cross-link more than 0.8 mol of NEM-S1 (IATR-S1)/mol of actin (Figs. 7 and 8) and the sum of cross-linked NEM-S1 and rigor-bound unmodified S1 was close to 1 (Table 3). The results of EDC-cross-linking of rigor actomyosin, performed in other laboratories, support a binding stoichiometry of 1:1, although this stoichiometry could be explained either by binding of one myosin head to one actin monomer (29) or by simultaneous binding of two heads with two monomers (17).

In the primary structure of actin, actin-actin and actin-myosin binding sites are separated (18, 30), but they are not independent. It has been shown that structural changes in the monomers accompanying polymerization affect their interaction with myosin heads and that the binding of S1 to actin monomers changes their structure and results in polymerization (31–33). This suggests that the effects of cross-

linking NEM-S1 (or IATR-S1) to myosin-binding regions of actin could be transferred across the monomer and affect the bonds with neighboring monomers. However, since the effect of myosin heads on the regions of monomer-monomer contact is not direct, the changes probably differ from those induced by EDC-cross-linking, which could explain the slightly different effects of the two actin modifications on the apparent K_m (Fig. 5 and Table 4).

The present work extends our previous studies on the sliding of random and block copolymers of unmodified actin with two kinds of differently modified monomers, cross-linked with glutaraldehyde and EDC (5). The previous study showed that the interaction of myosin heads with actin, which is necessary for the generation of sliding movement, involves only a limited number of monomers within one filament. Direct demonstration that blocking of up to 50% of myosin binding sites on actin with covalently cross-linked NEM-S1 (Fig. 8) does not affect movement strongly supports this conclusion.

Blocking of myosin binding sites on actin by covalent attachment of S1 had an additional advantage over EDC treatment: we could visualize the distribution of monomers unable to interact with myosin along individual filaments under an electron microscope. Analysis of the relationship between the sliding speed and the actomyosin ATPase cycle (34) led to the conclusion that the myosin head can translate an actin filament more than 100 nm by undergoing many active cyclic interactions with actin during one ATPase cycle. However, it was unclear whether cycling myosin heads remain associated with actin filaments throughout the ATPase cycle. Since the electronmicrographs of cross-linked acto-NEM-S1 complexes containing from about 0.1–0.5 mol of NEM-S1/mole of actin, where more than 60% of all filaments are sliding, show more than one head per every 100-nm segment of actin filament (Fig. 1), it seems likely that one myosin head cannot slide by 100 nm or more without several dissociation-reassociation cycles. Rapid (submillisecond) dissociation and reassociation during the active interaction of actin and myosin is consistent with the speed-dependent stiffness observed in contracting muscle fiber (35) and with the submillisecond rotational motion observed from S1 bound to actin in solution (36) and in active myofibrils (Ref. 37, in press).

It is intriguing that filaments containing more than 70% modified monomers are not motile (Figs. 7 and 8). In these filaments about 30% of the monomers (120 monomers/1-μm filament) remain free to interact with myosin. These filaments still activate HMM ATPase in solution and attach to the HMM-coated glass surface, confirming that they have sufficient unoccupied monomers to interact with myosin heads. It seems unlikely that these filaments would be nonmotile if movement were generated by the interaction of one cycling myosin head with only one monomer (38, 39); our results suggest that sliding movement requires the interaction of a cycling head with two or more monomers in sequence before it dissociates and reassociates again with another region of the filament. However, proof of this model requires coupling of the motility assay with electron microscopy for direct comparison of the amount and distribution of unoccupied monomers on individual motile and nonmotile filaments.

Although our results are consistent with the mechanisms of sliding postulating (*a*) cycles of association and reassociation of ATP-splitting myosin heads with sliding actin and (*b*) continuous interaction of cycling myosin head with a several monomers in sequence, several key questions remain unanswered. The fraction of monomers able to interact with myosin heads is not the only factor determining sliding velocity, since the effects were significantly different for the two methods of actin modification (Figs. 7 and 8). This suggests that the transfer of structural changes from the regions of intermonomer bonds to the myosin-binding sites on adjacent monomers involves a well defined path along the polypeptide chain of the actin monomer. Detailed structural and site-directed spectroscopic studies may be needed to test this hypothesis.

It is intriguing that we did not observe steric inhibition of sliding of cross-linked acto-NEM-S1 complexes, even where the amount of covalently bound NEM-S1 approached 0.5 mol/mol actin, as if cross-linked NEM-S1 and heads of glass-attached HMM always remained distant from each other. This raises the question of the relative positions of actin filaments and movement-generating heads—are filaments really "buried" between the heads, as suggested previously (34)? Since the actin-binding sites on myosin head are not on the tip of the head but near its center, this suggestion seems reasonable, but it was based on a low-resolution rotary-shadowed image of sliding filaments, not on direct measurement of the actin-myosin distance during sliding. If we assume that a certain degree of actin flexibility is necessary for correct positioning of myosin-binding sites on actin monomers relative to actin binding sites on cycling myosin heads, then sliding of cross-linked acto-NEM-S1 complexes with the same speed as unmodified actin becomes more understandable. Comparison of flexibility of the two types of modified actin filaments could help explain their different effects on sliding. The flexibility of an actin filament is related to its curvature as seen under the electron or optical microscope (40–42). The difference in the shape of copolymers (short and straight) and cross-linked complexes (long and curved) suggests that the complexes are more flexible than the copolymers. However, since even in the case of unmodified actin, short filaments are always straighter, the EM appearance of modified filaments can be regarded only as a suggestion, but not as sufficient evidence of their different flexibility. To verify this possibility, we are currently examining dynamic features of motile as well as nonmotile actin filaments using spectroscopic methods (43).

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